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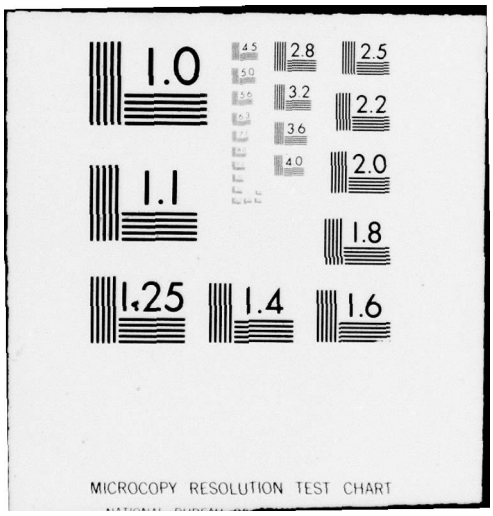
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DIRECT EXPOSURE OF MONOLAYERS OF MAMMALIAN CELLS TO AIRBORNE POLLUTANTS IN A UNIQUE CULTURE SYSTEM

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: AFOSR-TR-79-0958	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): DIRECT EXPOSURE OF MONOLAYERS OF MAMMALIAN CELLS TO AIRBORNE POLLUTANTS IN A UNIQUE CULTURE SYSTEM.	5. TYPE OF REPORT & PERIOD COVERED: Final Report, June 1, 1978-May 30, 1979 / 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): R.E. Rasmussen, T.T. Crocker and G.S. Samuelsen, M.E. Witte, J.T. Taylor, D.L. Swedberg	8. CONTRACT OR GRANT NUMBER(s): AFOSR-77-3343
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Community and Environmental Medicine, and School of Engineering, University of California, Irvine, Irvine, California 92717	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102F / 2312/A5
11. CONTROLLING OFFICE NAME AND ADDRESS: AFOSR, Building 410, Bolling AFB, (NL) Washington, D.C. 20332	12. REPORT DATE: August 1979 / 13. NUMBER OF PAGES: 38
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified / 15a. DECLASSIFICATION DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

Final rept. 1 Jun 78—30 May 79,

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

11 Aug 79

18. SUPPLEMENTARY NOTES

Ronald E. /Rasmussen, T. Timothy /Crocker, G. Scott /Samuelsen, M. E. /Witte J. T. /Taylor

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Mammalian respiratory cells
Nitrogen dioxide
ozone
Cytotoxicity
Mutagenesis
Airborne pollutants
Hydrazine

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A mammalian cell culture and exposure system has been developed that allows direct exposure of living cells to atmospheres containing pollutants such as oxidant gases and hydrocarbon vapors. The system is being used to detect cytotoxic effects of various airborne materials as well as their possible mutagenic effects. Oxidant gases include nitrogen dioxide and ozone; other materials include hydrazine, aircraft fuels, and known mutagens such as ethylmethane sulfonate and ethylene oxide. The goals of the project are to detect possible toxic effects of airborne materials and to determine whether

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synergistic effects may be produced in mixtures of materials found in the environment. Methods have been developed for the small-scale generation of atmospheres containing known mutagens and test materials including hydrazine and its derivatives and common aircraft fuels.

Tests for mutagenic effects of gaseous nitrogen dioxide on cultures of hamster lung cells have been negative at concentrations up to 10 ppm in air.

SUMMARY

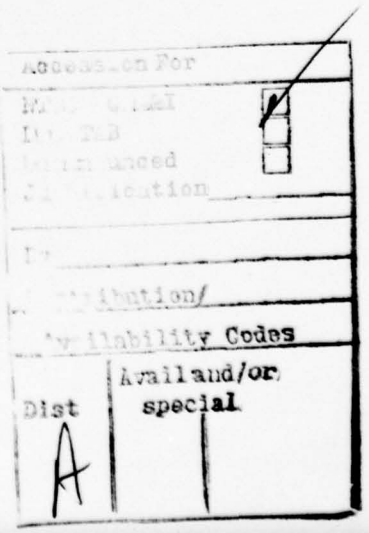
A cell culture and exposure system which allows nearly direct exposure of living cells to airborne pollutants has been developed and is presently being used for the exposure of cells to NO_2, ozone, and various other materials including hydrazines and hydrocarbon vapors. The details of the development and construction of the system have been described in previous reports. During the past year, the system has been modified and relocated to provide for more efficient operation. Exposure chambers and cell culture holders have been fabricated from stainless steel in order to permit exposure of the cultures to organic vapor atmospheres. Tests have shown that the cells being used in these studies (Chinese hamster and human lung-derived cell lines) are not affected by the presence of the stainless steel.

Methods have been developed for the generation, containment and monitoring of atmospheres containing known mutagenic chemicals, and for the exposure of cell cultures to these vapors. Atmospheres have been generated that contain hydrazine, or ethylmethane sulfonate, a mutagen that has been widely used in experimental studies of mammalian cell mutagenesis.

Tests for the direct mutagenic action of atmospheres containing NO_2 have been done using concentrations of up to 10 ppm. Although significant cell killing was seen, there was no evidence that NO_2 was mutagenic for mammalian cells. Studies are continuing that will include exposure to NO_2 in combination with other materials such as known mutagens and hydrocarbons.

Direct exposure of cells to ozone at 0.05 ppm has shown that this gas is significantly more cytotoxic than NO_2 by a factor of 3-5. Tests for mutagenesis by ozone have not been completed.

Primary cultures of cells from fetal rat lung have been grown on Millipore filters and examined microscopically. In these cultures the various cell types tended to reassociate and to form structures (e.g., ciliated epithelium, vascular structures) that resembled those found in vivo. This observation indicates that respiratory epithelial cell layers can be prepared and be exposed to various atmospheres in vitro.



PREFACE

This is the second annual report submitted under Grant No. AFOSR-77-3343. It describes progress in the development of a unique culture system for mammalian cells which allows direct exposure of the cells to air pollutants at realistic concentrations. This project is a collaborative effort of the Department of Community and Environmental Medicine and the School of Engineering, University of California, Irvine. Project monitor was William O. Berry, Ph.D., of the Life Sciences Directorate, AFOSR.

Principal investigator was Ronald E. Rasmussen, Ph.D., Associate Adjunct Professor Department of Community and Environmental Medicine. Co-principal investigators were T. Timothy Crocker, M.D., Professor and Chairman, Community and Environmental Medicine, and G. Scott Samuelsen, Ph.D., Associate Professor, School of Engineering, University of California, Irvine.

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SECTION I

CELL CULTURE EXPOSURE SYSTEM

Introduction

The experimental cell exposure system described in this report was developed with the goal of providing an in vitro model of the situation which obtains at the surface of the respiratory epithelium in vivo when airborne pollutants are encountered. The unique feature of the system is that mammalian cells are maintained in a viable state while at the same time being in nearly direct contact with the ambient atmosphere. This arrangement offers the possibility for detecting previously unknown effects of airborne materials, including cytotoxicity and mutagenic effects. The system is especially well suited for the examination of the biological effects of mixtures of gases.

The details of methods and materials for the presently-used 6 chamber exposure system were described in the annual report for 1977-78.

A major event in the present year was the relocation of the cell exposure system from the School of Engineering to a newly-renovated laboratory in the department of Community and Environmental Medicine. This activity offered the opportunity to evaluate the operation of the system and to incorporate changes as described below which will increase the efficiency of the system. Additional advantages have been gained by this relocation in that the necessary support facilities for cell culture are now in the same laboratory as the exposure system. The relocation has recently been completed and cell exposure experiments are in progress which will be described in subsequent reports.

In preparation for studies with organic vapors such as aircraft fuels, cell culture holders and exposure chambers have been fabricated from stainless steel. These materials have been evaluated in preliminary studies described below.

For the detection of potential cytotoxic, mutagenic, and possible carcinogenic effects of airborne, normally nongaseous, pollutants, it is a necessary requirement that reliable methods be at hand for generation, containment, and measurement of toxic mixtures.

Perhaps the first step in this regard is the development of such methods using an appropriate positive control substance against which test materials can be compared. Studies are described below in which methods have been explored for vaporization and monitoring of hydrazine and mutagenic hydrocarbons.

Cell culture studies during the present year are covered in Section II.

Relocation of Cell Exposure System

The relocation of the cell exposure system at this time was in part undertaken in order to take advantage of additional laboratory space which became available in the department of Community and Environmental Medicine subsequent to the completion of a new medical sciences research building. An additional reason was that the developmental stage, which required substantial input from Dr. G.S. Samuelsen and his staff of the School of Engineering, was complete, and it was considered that the space in the latter school should be used in projects more directly related to teaching and research in Engineering.

The new laboratory consists of a 20 ft. x 30 ft. room. In addition to the cell exposure system, permanent equipment includes a fume hood, an autoclave, 2 refrigerators, and 2 sterilizing ovens.

Modification of Exposure System

The exposure capacity of the system remains as previously described. The conversion to smaller exposure chambers has made it possible to place 2 chambers in each incubator, rather than one as previously, Figure 1 shows the arrangement of the exposure chambers within the incubator. There are 3 such incubators at present, with capability for exposure to NO_2 or O_3 or mixtures of the 2 gases.

The uniqueness of the cell exposure system depends on the ability to maintain viable cells in nearly direct contact with the test atmospheres without allowing the cells to dry out. This requires a careful balance between the rate at which the nutrient medium is perfused through the Millipore filter which supports the cells and the rate at which the

FIGURE 1. Lexan cell exposure chambers enclosed in 37° incubator for use with NO_2 and Ozone. Ozone generator is at the right rear of the incubator chamber. Carrier gas (5% CO_2:95% air) mixed with NO_2 enters through the coil of stainless steel tubing at the top of the chamber. Culture medium enters via tubing through a port in the righthand wall.

medium is removed from the exposed cells. Tests of various methods to accomplish the desired balance included vacuum systems for removing the medium, tilting the filter holders to allow the medium to run off, and controlled pumping with either a peristaltic or syringe-type pump. It was established that the best method was to use a multichannel peristaltic pump that would allow controlled, positive removal of medium from each cell filter holder. Two such pumps have been obtained. One is a model 1210 Harvard Instruments pump with up to 20 channel capacity and the other a Technicon Model II proportioning pump with capacity for 30 channels. The latter pump is on loan from AMRL at WPAFB, Ohio. Both pumps work quite well and are in use in the NO_2-O_3 exposure system and the organic vapor exposure system which is being developed.

Generation and Monitoring of Organic Vapor Pollutants

The cell exposure chambers for use with gases such as NO_2 and O_3 were made of Lexan plastic (General Electric Co.) as were the cell filter holders. This material would not be suitable for use with organic materials because of the solvent properties of the latter. In the past year stainless steel cell filter holders and exposure chambers lined with stainless steel foil have been fabricated. The filter holders were patterned on the Lexan holders previously used. Figure 2 shows one of the stainless steel filter holders disassembled, and Figure 3 shows two stainless steel filter holders which have been prepared with cell-bearing filters for exposure. The elbow connectors attached to the bottom section of the holders are for connection to the medium perfusion pump. The small tubes projecting from the top of the holder are connected to the peristaltic pump used for the removal of medium perfusing through the filters. For exposure to test atmospheres, the filter holders are placed upright inside the exposure chamber. Figure 4 is a photograph of a stainless steel lined exposure chamber with 4 cell filter holders in position. In Figure 5, the chamber door is in place and sealed with a silicone rubber gasket. The test atmosphere enters the left end of the chamber and exits at the right where it is trapped

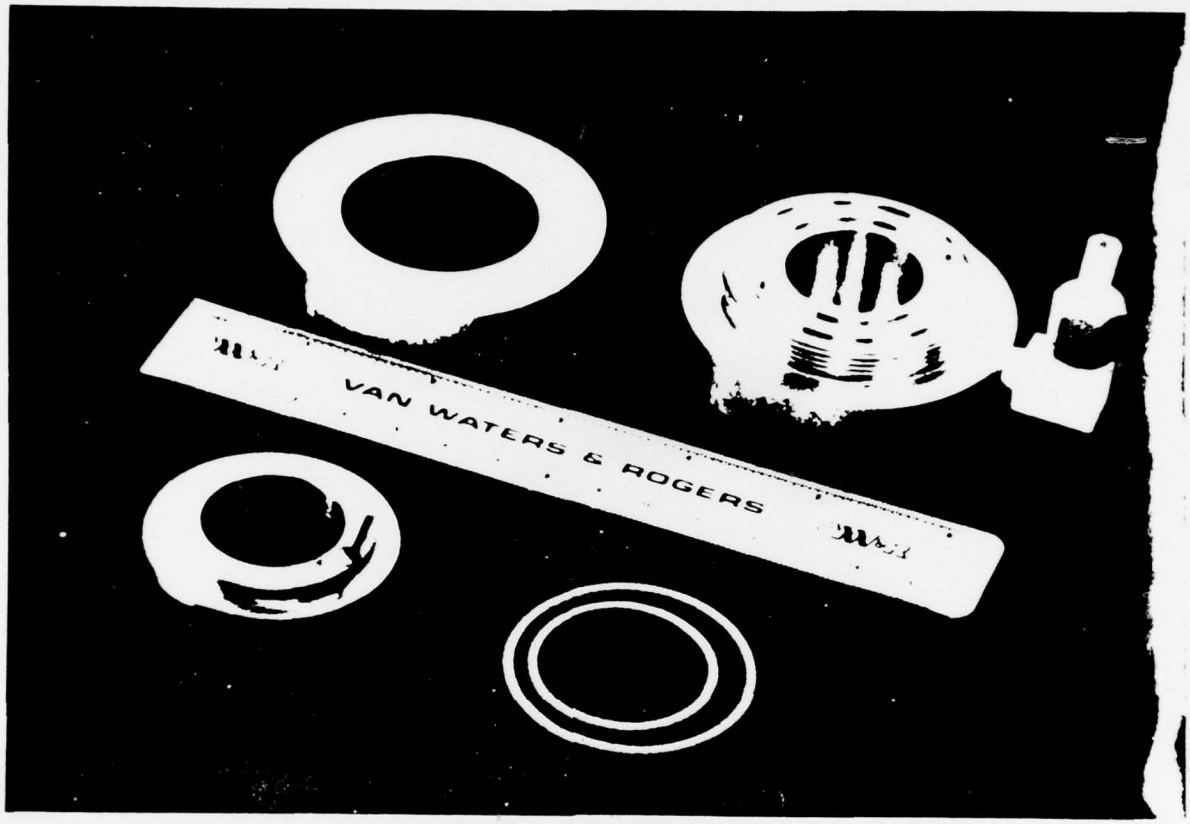

Figure 2. Stainless steel cell filter holder shown disassembled. The concentric "O" rings rest in the grooves in the base section (upper right). The filter with cells attached is placed atop the "O" rings and held in place by the retaining ring (lower left). The threaded cap (upper left) is then screwed into place effecting a seal between the filter and the "O" rings. The short projection on the retaining ring is a tube which permits removal of the overlaying growth medium from atop the cells.

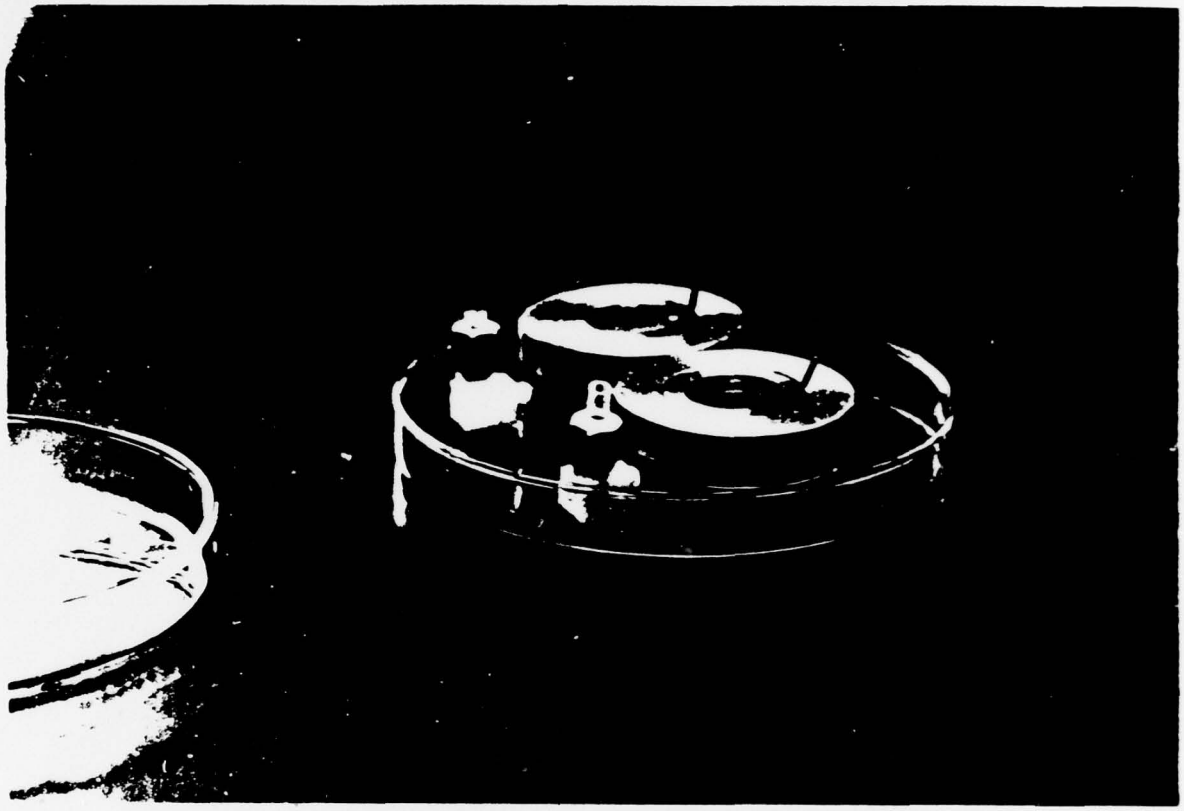

FIGURE 3. Stainless steel filter holders assembled with filters in place ready for placement in the gas exposure chamber.

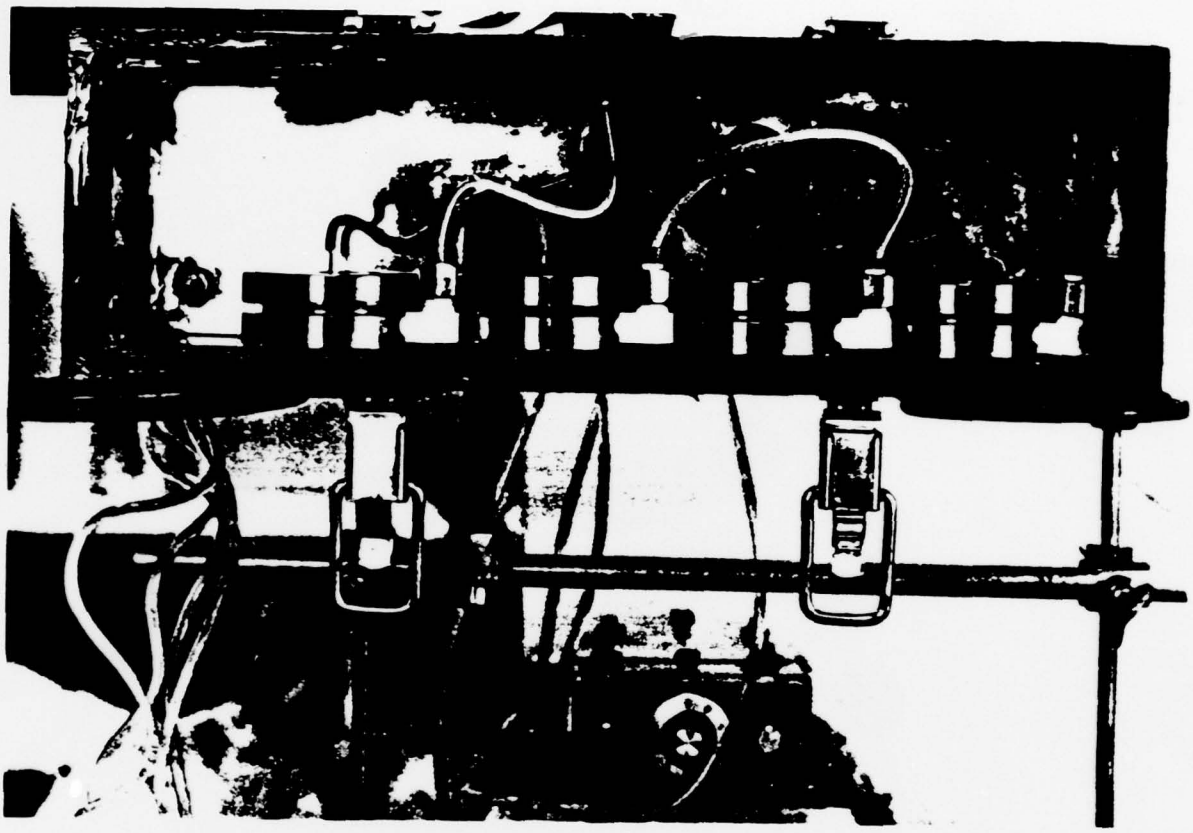

FIGURE 4. Stainless steel cell filter holders in place inside a stainless steel lined gas exposure chamber. Nutrient cell growth medium enters through tubes at the top of the chamber. Medium which perfuses through the filters is drawn off through the tubes at the rear wall of the chamber. The test gas enters at the left end of the chamber and exits at the right end.

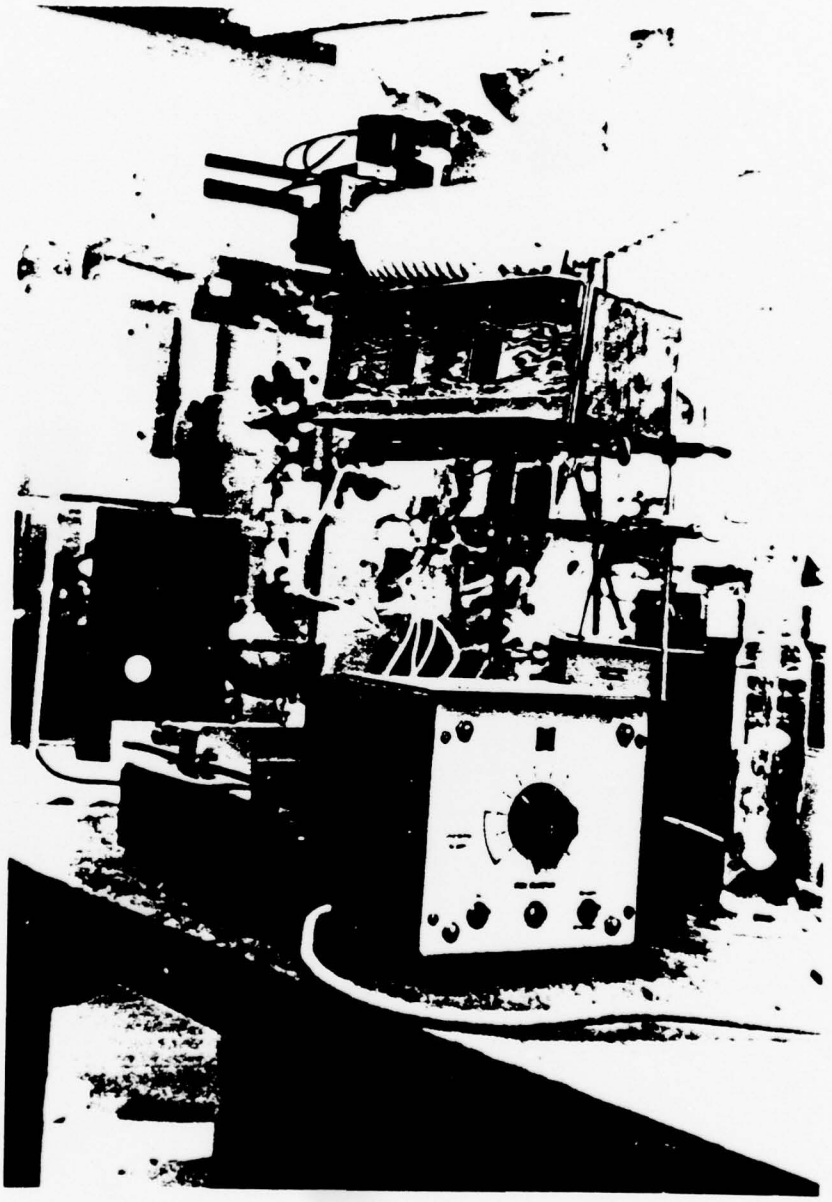

FIGURE 5. Gas exposure chamber with cover in place. The cover is sealed with a silicone rubber gasket. There are 2 glass windows to permit observation of the cell cultures during exposure. The syringe pump in the foreground provides nutrient medium to the cell cultures, and the peristaltic pump at the right rear draws off the medium which perfuses through the filters. The gas mixture is generated inside the plastic hood at the left rear (see Figures 6 and 7).

in a series of gas washing bottles (see below). Exposure of the cell layer to the test atmosphere is initiated by removing the overlaying medium using the peristaltic pump seen at the right rear of Figure 5, while fresh medium is provided to the filter holders by the syringe pump in the foreground.

Figure 6 is a diagram of the generation and containment system that is presently used in the development of methods to produce atmospheres containing mutagenic substances. This arrangement was designed in consultation with the Environmental Health and Safety Office at U.C. Irvine. The carrier gas (usually 5% CO_2:95% air) enters at the left through a glass wool filter. After passing through a rotameter the carrier gas passes through a Purafil column to remove traces of oxidant gases, then through a 0.47 um pore Millipore filter to remove particulates, and then into a mixing chamber where the test substance is vaporized by controlled heating. The amount of test material fed to the mixing chamber is controlled by Sage syringe pump. The mixture then enters the cell exposure chamber. At the outlet from the exposure chamber, a series of gas washing bottles is used to trap the mutagen. For example, in the case of ethylmethane sulfonate (EMS), the first 2 bottles contain 500 ml each of 0.001 N HCl, the 3rd bottle 500 ml of 1% sodium hypochlorite (Clorox), the 4th bottle 500 ml of water, and the 5th bottle is empty to provide a water drop-out before the gas stream passes through the final rotameter. It has been found that at a gas flow rate of 500 cc/min, over 90% of the EMS will be trapped in the 1st bottle.

An important point is that the gas mixture is pulled through the system by a vacuum at the exhaust end. In this way a negative pressure gradient is maintained from the outside to the inside of the system so that there is little chance of toxic materials escaping into the laboratory air. The syringe pump for the test material as well as the vaporization and mixing chamber are enclosed in a specially constructed hood made of acrylic plastic which is vented to the fume hood exhaust in the laboratory. A photograph of this special hood is shown in Figure 7.

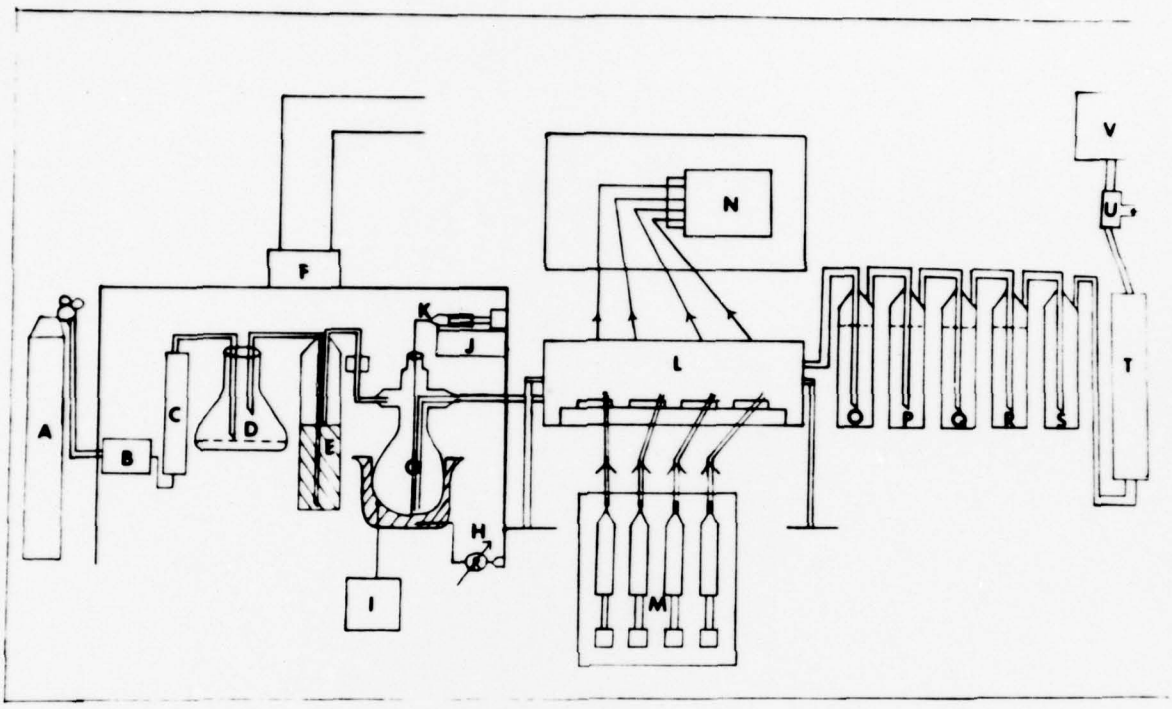

Figure 6. Generation and exposure system for atmospheres containing mutagenic chemicals. The system shown is in use for producing atmospheres containing ethylmethane sulfonate. A, carrier gas cylinder; B, glass wool filter; C, rotameter; D, flask with water to produce moderately humid atmosphere for activation of Purafil; E, Purafil column; F, vent fan for fume hood; G, vaporization and mixing chamber; H, rheostat for heating mantle; I, thermometer; J, syringe pump; K, capillary tube for introducing mutagenic chemicals into the mixing chamber; L, cell exposure chamber; M, syringe pump for cell growth medium; N, peristaltic pump for removal of cell growth medium from cell surface; O-S, gas washing bottles for trapping mutagenic vapors; T, rotameter; U, coarse adjustment for vacuum; V, vacuum pump.

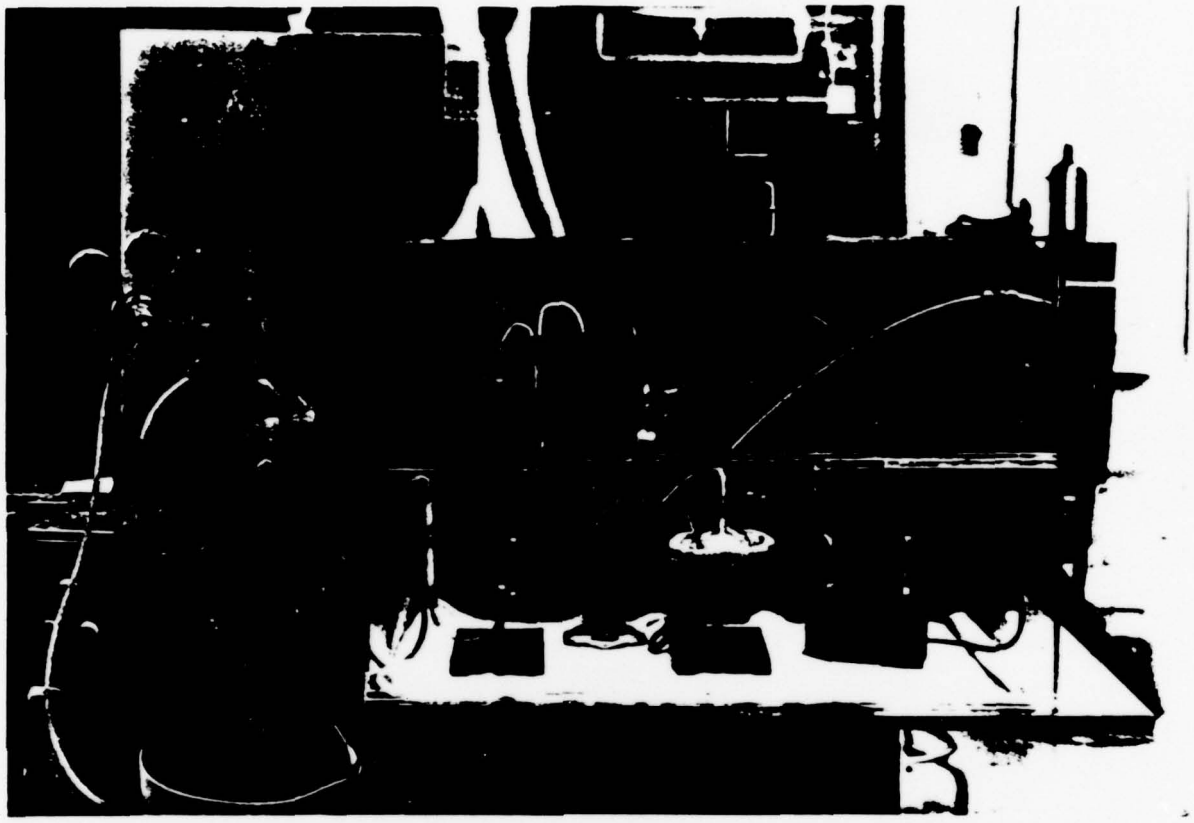

FIGURE 7. Acrylic plastic hood for containment of mutagenic vapor generation system. Carrier gas enters at the left through a filter and rotameter, and through the Purafil column into the mixing and vaporization chamber resting in the heating mantle at center. The syringe pump controlling the entry of the mutagen into the mixing chamber is at right. The gas mixture is conveyed to the exposure chamber via the Teflon tube seen leaving the hood at upper right.

Selection of Positive Control Substance

The criteria for positive control substance are:

That it is a proven mutagen for mammalian cells;

That it is a direct-acting mutagen, not requiring metabolic activation;

That it is readily vaporized in its active form;

That it is readily recovered and assayed from the vapor state;

That the vaporization and recovery can be controlled in a precise manner.

A number of known mutagens are being evaluated for potential positive control substances. They include ethylmethane sulfonate (EMS), ethylene oxide (ETO) and acrylonitrile (ACN). Each of these substances has its advantages and drawbacks. For example, EMS is a potent mutagen in cell cultures when it is dissolved in the culture medium. However, it has a relatively high boiling point and even though it can be vaporized without loss of activity, it tends to condense on cool surfaces. This is an advantage in that EMS vapors can be easily trapped in water in conventional gas washing bottles. However, it is difficult to maintain EMS in the vapor state during exposure of the cell cultures.

Ethylene oxide (ETO) is attractive as a positive control. Its low boiling point ensures that it would remain as a vapor during exposure, and it can be trapped and assayed in the same manner as EMS. We have prepared Mylar gas bags of approximately 50 liter capacity in which ETO; air mixtures will be prepared, and which can then be used as reservoirs to provide gas to the cell exposure chambers. These gas bags are patterned on those used at the THRU inhalation facility at WPAFB as reservoirs for hydrazine standards.

Assay Methods for Alkylating Mutagenic Chemicals

It is fortunate that most direct-acting alkylating agents can be assayed by colorimetric reactions.[1] The vaporized agents can be collected in dilute acid (e.g. 0.001 N HCl) with

little loss of activity. An aliquot of this solution is then assayed for the agent. As an example, Figure 8 is a standard curve prepared from solutions of EMS in 0.001 N HCl which shows a linear dependence of absorbance on concentration. The assay conditions for EMS were established empirically using the reported methods for other alkylating agents as a starting point. Table 1 summarizes the general procedure. Using this method, color reactions are produced with ethylene oxide and acrylonitrile as well as EMS.

TABLE 1

PROCEDURE FOR ASSAYING ALKYLATING AGENTS IN AQUEOUS SOLUTION

Reagents: 4-(p-nitrobenzyl)pyridine, 5% in acetone. (4NP).

K_2CO_3, 1 M

Buffer: Clark & Lubs, 0.1 M potassium biphthalate, pH 4.0

Acetone, reagent grade.

Procedure: To 3 ml of an unknown solution of alkylating agent having a concentration of 10^{-3} to 10^{-5} add 1 ml of 4NP and 1 ml buffer. Mix well and heat in boiling water bath for 20-30 min. Chill in ice. Blank contains 3 ml water in place of the alkylating agent solution. Tubes are read one at a time. To individual tube add quickly 4 ml acetone and 1 ml K_2CO_3. Mix well and read absorbance at 600 nm within 15 sec. with dist. water as absorbance blank. Subtract value obtained with reagent blank from readings. Standard curve must be determined empirically for agents with unknown properties. For EMS, the standard curve is prepared using concentrations between 10^{-5} to 10^{-3} M.

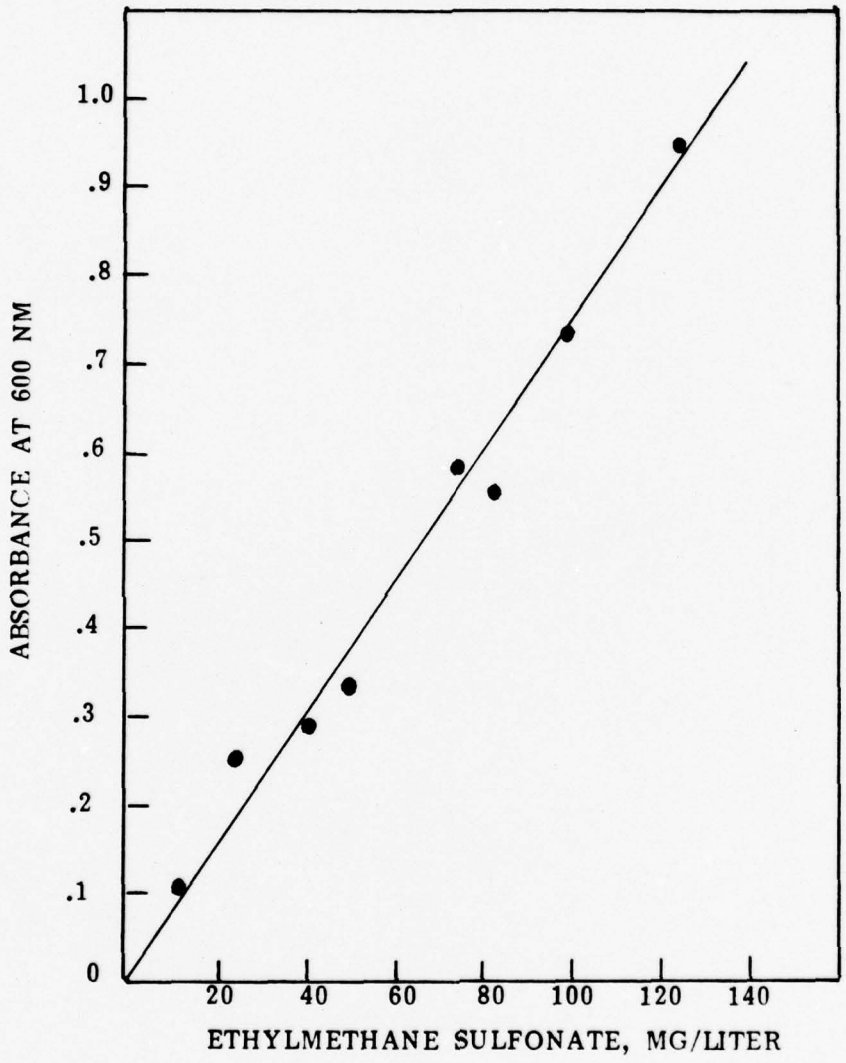

FIGURE 8. Standard curve for the determination of EMS in water. The assay was carried out as described in Table 1.

Generation and Assay of Hydrazine Atmospheres

Hydrazine (N_2H_4) and its derivatives of most immediate interest, Methylhydrazine (MMH), 1,1,-dimethylhydrazine (UDMH), and 1,2-dimethylhydrazine (SDMH) all have relatively low boiling points with hydrazine having the highest at 113°. In consultation with scientists at the THRU, WPAFB, Ohio, we have developed methods for the small scale generation and monitoring of hydrazine-containing atmospheres. The vapor generation and exposure system described above for EMS is, with minor modification, also suitable for the hydrazines. There are 2 major differences in the system for exposure of cell cultures to hydrazine vapor. The first is the materials that will come into contact with the hydrazine. Consultation with other laboratories[2] indicated that stainless steel (or other metals) is not compatible with hydrazine vapor, but plastic materials (e.g., polyethylene) are. Consequently, the Lexan chambers and filter holders will be used with hydrazines. The second difference will be in the vaporization method. A simple method has been developed for vaporization of hydrazine and mixing it with the the carrier gas stream to the cell exposure chamber. A Pyrex "T" replaces the mixing chamber shown in Figures 6 and 7. Liquid hydrazine is introduced into the side arm of the "T" via a 20 gauge spinal needle in such a way that the orifice of the needle is in the approximate center of the junction of the 3 arms of the "T". The rate of injection of the hydrazine is controlled by a syringe pump. Heat is gently applied to the "T" with an infrared heat lamp controlled by a rheostat. Carrier gas glows through the "T" junction, picks up the hydrazine vapor, and carries it to the cell exposure chamber. After leaving the cell exposure chamber, the gas can be trapped in dilute acid as in Figure 6, or directed to a continuous assay system. The latter system is patterned on the one developed at the THRU, WPAFB, Ohio, which has been used in animal exposure studies. For short-term exposures, the most convenient method is to trap the gas mixture in diluted acid, and

then to analyse an aliquot for hydrazine. We have applied 2 methods for quantitation of hydrazine in aqueous solution. The most readily applied is a colorimetric assay based on the reaction of hydrazines with dimethylaminobenzaldehyde. The sensitivity range is from about 20 ug/l to 3 mg/l. The assay procedure is summarized in Table 2. A second assay, dependent on the reduction of I_2, can be used as a continuous assay for hydrazine. The major advantages of this method are that it provides a method for continuously monitoring the hydrazine concentration, and it is particularly suited to long-term exposures. The details of the system developed at the THRU have been described previously in reports from that group.[3] The equipment required for the continuous assay includes a strip chart recorder, a flow cell spectrophotometer, and a peristaltic pump. These items have been provided to us on loan from the THRU.

TABLE 2

DETERMINATION OF HYDRAZINE WITH DIMETHYLAMINOBENZALDEHYDE

Reagents: Dimethylaminobenzaldehyde, 0.8 g dissolved in 40 ml abs. Ethanol plus 4 ml Conc. HCl.

HCl, 1 N

N_2H_4, 1 mg/ml in 0.1 N HCl, diluted to give concentrations for standard determination of 0.25-10 mg/l.

Procedure: To 1.0 ml of N_2H_4 solution add 4.0 ml of DMAB reagent and 5 ml of 1 N HCl. Mix well and allow to stand at room temperature for 30 min. Read absorbance at 458 nm.

SECTION II

CELL CULTURE STUDIES

Introduction

Goals of the cell culture studies during the past year have been:

To test for the mutagenic effect of oxidant gases on mammalian cells;

To further study the mechanism of cytotoxic action of NO_2;

To determine the effect of NO_2 on DNA synthesis;

To explore methods for establishing cultures of respiratory cells;

To test for possible synergistic effects of NO_2 and O_3;

To test synergistic effects of oxidant gases on chemical mutagenesis.

Studies concerned with the latter 2 points have of necessity been postponed until the current year. The reasons for this have been the down-time of the exposure system during the relocation and manufacturer's delays in being able to supply the Harvard Instruments peristaltic pump. These investigations have been resumed now that the exposure system is again in operation.

Test for Mutagenic Effect of NO_2

Methods

To provide the best chance of detection of mutagenic effects of NO_2 it was decided to expose a large number of cells to a relatively high concentration of NO_2. Cultures of line V-79 Chinese hamster lung fibroblasts were prepared in roller bottles fitted with special caps to allow flushing of the bottles with NO_2 during incubation of the cell cultures. The surface area available for growth was approximately 700 cm^2, and the volume of medium (approximately 50 ml) covered about 10% of the cell layer. At the time of exposure to NO_2, the bottles contained about 1×10^7 cells, and were rotating at 1 revolution every 2 minutes. The NO_2 concentration was 10 ppm, control bottles

were flushed with clean air, and the total exposure period was 4 hours. At the end of the exposure, the cells were harvested from the bottles with trypsin, counted, and aliquots plated to determine the viability of the cells recovered. The remaining cells were then seeded into culture flasks (75 cm^2) at approximately 10^6 cells per flask, and subcultured at intervals when the cell layer in the flasks became confluent. The subculturing was continued for 10 days to allow sufficient time for mutation expression.[4]

To test for the presence of mutant cells, 10^5 cells were seeded into 100 mm plastic dishes (10 dishes each for NO_2-exposed and control) containing either medium with 30 ug/ml 6-thioguanine or 10^{-3}M ouabain. After incubation for 10-12 days, the plates were stained and resistant colonies counted.

Experimental Results

Previous studies had established that NO_2 has a strong cytotoxic effect on V-79 cells, even at levels as low as 0.15 ppm. In this test for mutagenesis by NO_2, substantial cell killing was expected and was observed. When at the end of the NO_2 exposure it was attempted to recover the cells by trypsinization, the NO_2-exposed cells congealed into a viscous mass, probably as the result of cell lysis and release of DNA. Of those cells recovered, only about 15% were able to form colonies when seeded into dishes. Essentially all of the cells exposed to clean air were recovered, and they showed a colony-forming ability of about 65%, which is similar to unexposed control cultures. Challenge with either 6-thioguanine or ouabain indicated that the NO_2 exposure did not induce the formation of mutants among the surviving cells. These data are summarized in Table 3, following.

TABLE 3

TEST FOR MUTATION OF V-79 CELLS BY 10 PPM OF NO_2

# of Cells at Start of Exposure	# of Cells Recovered	Colony forming Ability (%)	6-Thioguanine Resistant per 10^5 Survivors	Ouabain resistant per 10^5 Survivors
Clean Air: 10^7	1.34×10^7	63.5	15±5	6±3
NO_2: 10^7	3.28×10^6	17.7	12±7	4±2

Previously reported experiments using ethylmethane sulfonate at 10^{-2} M for 2 hours as a mutagen produced a yield of 39±7 6-thioguanine resistant mutants per 10^5 survivors (1977-78 Annual Report). We conclude that under these conditions of exposure, NO_2 is not mutagenic for this cell line. Tests for the mutagenic effect of NO_2 were also conducted at lower NO_2 concentrations of 0.15 and 1.0 ppm. In neither case was there any indication of mutagenesis, and only slight cell killing at 1.0 ppm in the roller bottle cultures.

Effects of NO_2 on Cell Morphology

Methods

Previous studies (1977-78 Annual Report) had shown that when V-79 cells were planted on filters as widely dispersed cells and then exposed to 0.15 ppm of NO_2, only about 10% of the cells remained able to form colonies after a 6-hour exposure. Further studies with radiolabeled cells suggested that cells were being lost from the filters either as the result of detachment from the filter or disintegration of the cells. In order to obtain further information on the cytotoxic effects of NO_2, we have examined cells immediately following exposure to NO_2 at 0.15 ppm. Line V-79 cells were seeded onto Millipore filters (10^4

cells per filter) as previously described, and allowed to attach overnight. The filters were then assembled into plastic filter holders and exposed for 4 hours to NO_2 at 0.15 ppm. The filters were then removed from the holders, fixed in 70% ethanol, stained with hematoxylin, dried and mounted. Some filters were incubated in immersed culture for a further 24 hours before fixation.

Photomicrographs of representative areas were taken with a Polaroid attachment. Cell numbers per unit area were determined by counting the number of cells in randomly chosen microscopic fields, the areas of which were calibrated using a stage micrometer and an eyepiece grid.

Experimental Results.

Photomicrographs of NO_2-exposed and immersed control cultures of line V-79 cells are shown in Figure 9. The most obvious differences between the 2 cultures are that the NO_2-exposed cells appear rounded up and less firmly attached to the surface of the filter, and there is some indication of nuclear distortion or fragmentation compared to the immersed controls. Cells exposed to clean air (not shown) also showed some evidence of being less firmly attached, but did not show the nuclear effects.

Direct counts of the numbers of cells remaining on the filters following NO_2 exposure also indicates that cells were detaching. Table 4 summarizes the results of 2 experiments designed to investigate this possibility.

TABLE 4

EFFECT OF NO_2 ON THE NUMBER OF V-79 CELLS REMAINING ATTACHED TO FILTERS

Experiment #	Cells Planted per Filter	Treatment[a]	Cells per Filter at 24 hours Post-treatment
1	1×10^4	4 Hr Air	2.61×10^4
	"	4 Hr NO_2	1.41×10^4
	"	Imm. Cont.	4.30×10^4

Figure 9a

Figure 9b

Figure 9. Photomicrographs of V-79 cells grown on Millipore filters and a) held in immersed culture or b) exposed for 4 hr to 0.15 ppm of NO_2. At the conclusion of the exposure, the filters were fixed in 70% ethanol (10 min), 50% methanol (10 min), stained with Harris' hematoxylin, dehydrated, cleared and mounted in Permount. Magnification approximately 1000 times.

Experiment #	Cells Planted per Filter	Treatment[a]	Cells per Filter at 24 hours Post-treatment
2	3×10^4	4 Hr Air	2.62×10^4
	"	4 Hr NO_2	1.46×10^4
	"	Imm. Cont.	2.73×10^4

a. NO_2 concentration was 0.15 ppm; Imm. Cont. = Immersed control filter not exposed to air or NO_2.

Cytotoxic Effect of Ozone on V-79 Cells

Methods and Experimental Results

The cell culture methods for the ozone exposures were essentially the same as for the NO_2 exposures. The cells were allowed to settle onto the filters and allowed 4 hours for attachment. These experiments were done using the earlier exposure chambers of approximately 1 ft^3 volume. The gas flow rate was 4 liters/min and the ozone concentration was 0.05 ppm as continuously measured by a Dasibi monitor. Viability after exposure was estimated, as before, by the ability of cells remaining on the filters to form microscopic colonies upon subsequent incubation in submerged culture. Table 5 presents the results of this study. The experiments involving different lengths of exposure time were not done at the same time because of limitations in equipment. However, an effort was made to ensure that the cultures were at the same stage of growth in each experiment, and that the manipulations were done in the same way each time.

TABLE 5

CYTOTOXIC EFFECT OF OZONE ON V-79 CELLS[a]

Length of Exposure	Colonies on Immersed Filters	N/N_o	Colonies on O_3-Exposed	N/N_o	Colonies on Air-Exposed	N/N_o
1 Hr	59.1±7.2	0.30	55.8±9.4	0.28	59.2±15	0.30
2 Hr	56.2±13	0.28	39.2±22	0.20	61.5±5.3	0.31
3 Hr	59.1±7.2	0.30	33.8±20	0.17	55.8±13	0.28
4 Hr	71.2±16	0.36	45 ± 58	0.045	81 ± 37	0.40
6 Hr	63.5±8.7	0.32	122 ± 64	0.024	44 ± 32	0.22

a. All filters were planted with 200 cells/filter except for the 4 hr and 6 hr ozone exposed which were planted with 1000 and 5000 cells respectively. The values are the mean of 2-4 filters ± 1 standard deviation.

The results found with V-79 cells are in general agreement with previously reported results with other cell lines in that ozone seems to be somewhat more toxic than NO_3 by a factor 3-5. The present results are roughly equivalent to what would be found with NO_2 at 0.15 ppm for the same exposure times.

Effect of NO_2 and $NaNO_2$ on DNA Synthesis in V-79 Cells

Methods

NO_2 and $NaNO_2$ have been reported to be mutagenic in plants and bacteria.[5,6] Further, it has been demonstrated that nitrous acid formed under acidic conditions with $NaNO_2$ or NO_2 reacts with DNA bases in a way that could be mutagenic.[7] In a recent study it has been shown that many chemical and physical mutagens inhibit DNA synthesis in mammalian cells in culture in a characteristic way.[8] After a brief exposure to the mutagenic substance, the rate of DNA synthesis is inhibited to a certain degree, and the rate of DNA synthesis continues to decline for some additional time period, up to several hours after the short exposure to the mutagen. A possible interpretation of this observation

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is that the mutagen has introduced blocks to continuing synthesis into the cellular DNA or in some way has stopped the initiation of new DNA synthesis. Non mutagenic cellular toxins may inhibit DNA synthesis while they are present, but upon removal, normal DNA synthesis is soon restored.

Prior to exposure to the test material, cell cultures are prelabeled with ^{14}C-thymidine (0.01 uci/ml) for 24 hours. This procedure uniformly labels all of the DNA in the culture and provides a method of estimating the amount of DNA present during subsequent measurements. After treatment with the test material, sample cultures are pulse-labeled for 10 min with ^{3}H-thymidine (10 uci/ml) and the cells immediately harvested. At intervals after treatment, additional cultures are pulse-labeled and harvested in a similar manner. Measurement of $^{3}H/^{14}C$ ratio in the cells harvested at each sampling time provides an index of the rate of DNA synthesis at that time. In the studies reported here, V-79 cells were exposed to solutions of $NaNO_2$ at various pH values, or to gaseous NO_2 at 0.15 ppm. As a positive control, additional cultures were exposed to 10 J/m^2 of UV light at 254 nm.

Experimental Results

Table 6 summarizes the results obtained with UV light and 0.15 ppm of NO_2 on the inhibition of the rate of DNA synthesis in V-79 cells. The results are expressed as the ratio of the rate of DNA synthesis in the treated culture to that in the appropriate control at various times after treatment. The results with UV light are as expected. Immediately after exposure, there was only a slight difference between the UV-exposed cells and the control, but with increasing times after treatment, the rate of DNA synthesis in the UV-exposed cultures progressively declined compared to the control. This was not seen in the NO_2-exposed cultures. The rate of DNA synthesis was about 50% that of the control immediately following exposure, and remained at this level during the time that samples were taken. The conclusion is that NO_2 in the gaseous form does not directly interact with DNA to inhibit the rate of synthesis in the same way as many other mutagens.

TABLE 6

RATE OF DNA SYNTHESIS IN V-79 CELLS EXPOSED TO UV OR NO_2

UV-Exposed Cultures[a]			NO_2-Exposed Cultures[b]		
Time after UV(Hr)	UV/Control	Range	Time after NO_2(Hr)	NO_2/Control	Range
0	1.00	0.65-2.19	0	0.48	0.40-0.58
0.5	0.51	0.33-1.25	0.5	0.37	0.23-0.66
1.5	0.29	0.18-0.43	1.5	0.46	0.27-0.81
2.5	0.27	0.19-0.40	2.5	0.69	0.29-1.12
3.5	0.57	0.49-0.67	3.5	0.32	0.14-0.76
4.5	0.31	0.20-0.44			

a. Values are based on duplicate cultures. ^{14}C-DPM/culture ranged from 1300-2800; ^{3}H-DPM/culture ranged from 1000-7000.

b. Values are based on duplicate (0, 0.5, and 1.5 Hr) or triplicate (2.5, 3.5 Hr) cultures. ^{14}C-DPM/culture ranged from 1000-2700; ^{3}H-DPM/culture ranged from 300-2900.

A similar study was done with $NaNO_2$ at 10^{-3} M in phosphate-buffered saline (PBS) adjusted to 4 different pH values, 4.0, 5.0, 6.0, and 7.0. Cultures of V-79 cells prelabeled with ^{14}C-thymidine as described were exposed for 1 hour to the $NaNO_2$ solutions in PBS, and then returned to normal growth medium and pulse-labeled at intervals with ^{3}H-thymidine as before. Control cultures were exposed to PBS at the same pH values, but without $NaNO_2$. The results of this experiment are shown in Table 7.

TABLE 7

RATE OF DNA SYNTHESIS IN V-79 CELLS AS THE RATIO OF $NaNO_2$-EXPOSED TO CONTROL

Time after Treatment	pH 4.0	pH 5.0	pH 6.0	pH 7.0
0	0.108	0.040	0.640	1.70
1.5	0.221	0.144	0.627	1.40
3.0	0.326	0.273	0.488	1.34
4.0	0.218	0.181	0.338	1.10

Values are based on triplicate cultures. ^{14}C-DPM/culture ranged from 1100 to 5100; ^{3}H-DPM/culture ranged from 400 to 34,000.

The pronounced effect of the combination of low pH and $NaNO_2$ is very clear from this experiment. In the cultures exposed to PBS alone, there was significant depression of the rate of DNA synthesis only in the cultures exposed at pH 4.0, in which it was depressed to the extent of about 60% (data not shown). The rate of DNA synthesis in the PBS control cultures at pH 5.0 and 6.0 was not significantly different from that at pH 7.0. The greatest inhibition of DNA synthesis was at pH 5.0 with $NaNO_2$, where it was depressed to 5% of the control, and showed only slight recovery over the time studied. Also very interesting is the result seen at pH 6.0. An initial slight depression increased over the period of the experiment until at 4 hours, the rate of DNA synthesis was only 33% of the control. This result suggests that DNA damage may have occurred which resembles that produced by mutagenic chemicals or agents (e.g., UV light as above). A possible explanation is that nitroso compounds were formed intracellularly which then reacted with DNA. It has been reported that mutagenic nitroso compounds can be formed in the environment by reaction between naturally-occurring amines and nitrous acid.[9] We have confirmed these findings using methylguanidine in an acidified mixture with $NaNO_2$. However, we have not yet tested for possible mutagenesis at pH 6.0 by $NaNO_2$ under the conditions of the experiment described here.

A further interesting result is the stimulation of the rate of DNA synthesis by $NaNO_2$ at pH 7.0. It appears to be a real effect, but we have no explanation at this time. Studies of cell permeability or changes in enzyme activities have not been done.

This study suggests that the biological effects of NO_2 may be strongly pH dependent within the physiologically tolerable range (i.e., as opposed to the well-known chemistry at extremes of pH). Therefore, in future studies in which mixtures of oxidant gases and other test materials will be examined for their biological effects, the pH of the cellular milieu must be closely controlled.

Test for Toxic Effects of Stainless Steel Filter Holders

Methods

The possibility of cytotoxic effects of the new stainless steel cell filter holders was examined in 2 experiments using CHO (Chinese hamster ovary) cells as indicators. Six cell filter holders each of the plastic and stainless steel design were assembled complete with filters and nutrient medium in the lower chamber. A suspension containing 200 cells was then placed in the well above the filter and the assemblies placed in an incubator for 20 hours. At the end of this time, the filters were removed and placed in submerged culture for 6 days to allow surviving cells to form microscopic colonies. The filters were then fixed and stained with hematoxylin, and the colonies counted.

Experimental Results

Both experiments indicated that there was only slight, if any, cytotoxicity from the stainless steel holders. The results are shown in Table 8. The difference in absolute numbers of colonies in the 2 experiments may be due in part to experimental error in cell counting, and also due to the different growth media used in the experiments. In experiment 1, the medium was Eagle's minimal essential medium (MEM) which contains only the nutrients essential for cell growth. In experiment 2, the medium was Ham's F12 as modified by Kaighn (F12K) which is enriched with additional amino acids and growth factors.[10]

TABLE 8

SURVIVAL OF CHO CELLS IN STAINLESS STEEL FILTER HOLDERS

Expt. No.	St. Steel	Plastic	Petri Dish
1	100+10	116+19.4	124+10.8
2	197+14	209+14.5	184+13.6

Primary Culture of Respiratory Cells

Methods

One of the goals of this project has been to provide an in vitro model of the respiratory epithelium for use in the exposure system described above. Since the respiratory system is made up of so many types of cells, it is practically impossible using presently available methods, to separate and culture each individually. However, certain types can be isolated, and cultured which retain some features of their in vivo morphology and function. For example, alveolar macrophages can be flushed from the intact lung, and cells resembling type 2 alveolar cells can be isolated and cultured from dispersed lung tissue of rats.[11] The latter method involves removal of lung from young adult rats, mincing the tissue, and incubation of the mince with trypsin (0.25%) which releases some of the cells from the tissue. The dispersed cells are then seeded into petri dishes with enriched medium.[10] A small fraction of the cells will attach to the surface and begin to divide. After several days, the dishes are examned microscopically, and colonies of cells having the desired characteristics are selected from the dish and transferred to new dishes or culture flasks. It has been found that cell cultures established in this way will contain clones of cells that can be selected and maintained in culture for many passages.

Since the respiratory epithelium in vivo is multicellular with many cell types arranged in a characteristic way, it was not clear initially how this could be reproduced in vitro. A clue was given by a recent report[12] in which it was shown that when fetal rat lung cells were first dispersed enzymatically and then cultured in vitro, there was some indication of reassociation of cells into structures resembling those in the in vivo epithelium. We have carried out studies in order to determine whether it might be possible in this way to prepare sheets of cells on Millipore filters which would contain those cellular components and structures found in vivo. The lungs of near-term fetuses from Fisher 344 rats were aseptically removed and incubated with trypsin (0.25%, 30 min) to disperse the cells. The freed cells were collected by gentle centrifugation, suspended in growth

medium (F12K) and seeded onto washed, sterile, Millipore filters of the type used in the cell exposure experiments. The cell suspension was confined to a circular area of the filter approximately 20 mm in diameter. The cell number was estimated at 4-5 x 10^6 per filter, based on hemocytometer counts. This is a sufficient number of cells to make several monocellular layers in the available area. It was considered that this would encourage the cells to become closely associated, and to reestablish the intercellular relationships which they had in vivo. At intervals after planting, sample filters were fixed and embedded for sectioning. Some filters were stained and mounted whole after fixing.

Experimental Results

Two clones of cells (designated ARL-12 and ARL-14) presumed to have originated from type 2 lung cells have been isolated and have been in culture continuously since March, 1978. The cells have an epitheial growth habit, and contain cytoplasmic inclusions which have been characteristically associated with type 2 cells in vivo and in vitro.[10,11] Cell cycle time in vitro is about 24 hr, but this varies with the type of culture medium and the density of the culture. Planting efficiency (colony-forming ability) is about 10% when cells are seeded at a density of 200 cells per 60 mm dish. This is considered low for a continuous cell line. Karyotype analysis indicates that the cells are not diploid. Table 9 summarizes these findings. The 2n number for rats is 42.

TABLE 9

KARYOTYPE OF CELL STRAINS ARL-12 AND ARL-14 RAT LUNG CELLS

Chromosome Number	ARL-12	ARL-14
42	5	5
43	6	18
44	12	1
45	1	1
	24	25

Soon after the clones were isolated, representative cultures were preserved in liquid nitrogen for future reference. Experiments are presently in progress to evaluate the usefulness of these cell clones for their usefulness in the cell exposure system.

The results of the studies with the fetal rat lung cells have been encouraging. These studies established that the dispersed cells will in fact "sort themselves out" when they are seeded onto a Millipore filter in a restricted area. Photomicrographs of representative structures are shown in Figures 10 through 13. This experiment extended over a period of approximately one month, with sample filters examined periodically. It appeared that the maximum amount of in vitro "differentiation" was present at 10-12 days after preparation of the cultures. In later cultures, the differentiated structures shown in the Figures were not as numerous or obvious. This observation has not been quantified, but is based only on the examination of the surface of the fixed and stained filters. The Figures shown are all of cultures which had been maintained for 11 days in vitro. Figure 10 is a low-power (approximately 100 times) view of the surface of the cell sheet. Epitheloid and fibroblastic cells are evident. The dark nodules are masses of cells of unknown nature which protrude from the surface of the cell sheet. Figure 11 is a high power (approximately 1000 times) view of a cross section of one of the nodules. The multicellular nature is evident, as is the multilayered cell sheet that has formed on the surface of the filter which is at the bottom. In the body of the filter, near the base of the cell sheet are faintly staining protrusions from the cells into the pores of the filter.

Figure 12 is a cross section of a filter showing an area of ciliated epithelium, and again at the junction of cell and filter, there is an indication of cellular processes growing into the filter. Figure 13 is a cross section showing one of many vascular elements that were found in these preparations. In some cases the lumen appeared to be filled with possibly mucoid material, but its nature has not been confirmed.

Figure 10

Figure 10. Fetal rat lung cells on Millipore filter at 11 days in culture. Filters were fixed in Bouin's fixative, stained with hematoxylin, cleared and mounted in Permount. Magnification approximately 100 times.

Figure 11

Figure 11. Cross section of fetal rat lung cells on Millipore filter, 11 days in culture. Note multicellular nodule and multilayer cell sheet on filter. Fixed in Bouin's fixative, prestained with hematoxylin, embedded in paraffin, and sectioned at 5 um. Magnification approximately 1000 times.

Figure 12. Cross section of fetal rat lung cells on Millipore filter at 11 days in culture showing ciliated epithelial cells. Fixed and sectioned as in Figure 11. Magnification approximately 1000 times.

Figure 13

Figure 13. Cross section of fetal rat lung cells in culture on Millipore filter 11 days, showing example of prevalent vascular structure. Fixed and sectioned as in Figure 11. Magnification approximately 1000 times.

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